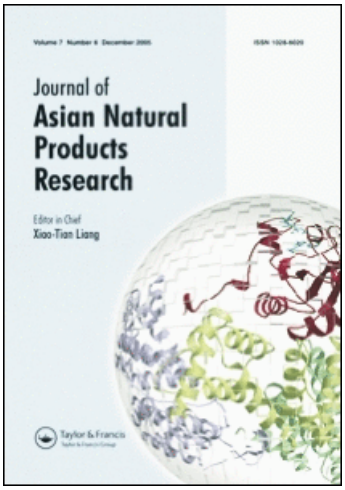

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Ginsenoside-Rg1 mediates microenvironment-dependent endothelial differentiation of human mesenchymal stem cells *in vitro*

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Ginsenoside-Rg1 mediates microenvironment-dependent endothelial differentiation of human mesenchymal stem cells *in vitro*

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Bone marrow-derived mesenchymal stem cells (MSCs) possess a multi-lineage differentiation potential and have the ability to repair and rebuild injured vessels. The autologous differentiated MSC transplantation also makes possible the tissue-engineered grafts. Therefore, the efficient endothelial differentiation of MSCs could be beneficial in the successful injured vessel repair and engraftment. Ginsenoside-Rg1, the most prevalent active constituent of ginseng, is a potent proangiogenic factor of vascular endothelial cells and also has the ability to enhance the proliferation of bone marrow cells. The aim of this study is to investigate the role of ginsenoside-Rg1 in the microenvironment-dependent endothelial differentiation of human MSCs (hMSCs) *in vitro*. The endothelial differentiation environment was established by co-culturing hMSCs with mature endothelial cells (human umbilical vein endothelial cells) indirectly *in vitro*. Reverse transcriptase-polymerase chain reaction analysis and fluorescence immunocytochemistry showed a strong expression of endothelial-specific markers such as CD31, Von Willebrand factor, and VE-cadherin. Electron microscopy showed the endothelial characteristic Weibel–Palade bodies of differentiated hMSCs. The increased expression of CD31 demonstrated that Rg1 promoted the endothelial differentiation of hMSCs. The findings here show the differentiation of hMSCs into cells with phenotypic features of endothelial cells using indirect co-culture with mature endothelial cells and provide the evidence that ginsenoside-Rg1 can promote the milieu-dependent endothelial differentiation of hMSCs *in vitro*.

Keywords: ginsenoside-Rg1; bone marrow cells; mesenchymal stem cells; cell differentiation; endothelial cells

1. Introduction

Endothelial impairment is the underlying pathological manifestation of many vascular diseases, such as hypertension, diabetes, coronary disease, and heart failure. Endothelial reconstruction can occur by migration and proliferation of surrounding mature endothelial cells. However, their capacity to substitute damaged endothelium is limited. Therefore, the endothelial repair may need the support of other cell types. Bone marrow-derived mesenchymal stem cells (MSCs) possess a multi-lineage differentiation potential [1] and have the capability to repair and rebuild various tissues [2–6]. Accumulating evidences suggested that MSCs have the ability to repair and rebuild injured

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vessels [7,8]. Angiogenesis, the formation of new blood vessels by sprouting from preexisting vessels and vascularization, the primitive vascular network formation from embryo angioblasts differentiated from mesodermal cells, is involved in the process of vascular repair and reconstruction. The endothelial differentiation of MSCs may promote the angiogenesis and vascularization. In addition, the autologously differentiated MSC transplantation makes possible the tissue-engineered grafts. Therefore, the efficient endothelial differentiation of MSCs could be beneficial in the successful injured vessel repair and engraftment.

Panax ginseng, used for over 1000 years in Chinese medication, has shown many clinical and pharmacological activities. Of the active components, the ginsenosides (commonly known as saponins) exhibit a variety of cardiovascular actions [9–11]. Of the most active saponins, ginsenoside-Rg1 (Figure 1) plays an important role in the protective effect on injured endothelium [12]. Rg1 also played a similar role as IL-3, IL-6, and G-CSF to enhance the proliferation of bone marrow cells [13].

The aim of this study is to investigate if ginsenoside-Rg1 could mediate the endothelial differentiation of human MSCs (hMSCs) *in vitro*. The endothelial differentiation environment was established by indirect co-culturing hMSCs with mature endothelial cells. Under these conditions, MSCs could express several features of mature endothelium, including the expression of CD31, Von Willebrand factor (VWF), and VE-cadherin. Moreover, ginsenoside-Rg1 can promote the milieu-dependent endothelial differentiation of hMSCs *in vitro*.

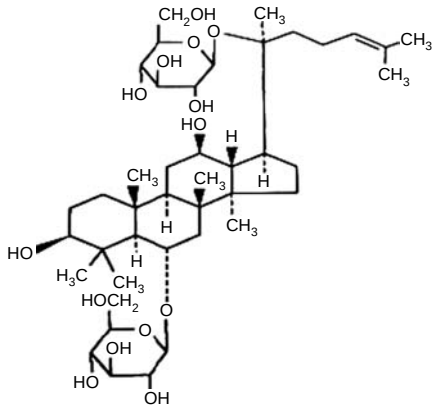

Figure 1. Chemical structure of ginsenoside-Rg1.

2. Results

2.1 Cell culture of hMSCs

Cells in passage 0 demonstrated a fibroblast-like, spindle-shaped morphology (Figure 2(A)). In later passages, the spindle-shaped cells began to display broadened, flat morphology. Endothelial experiments were performed on cells from P3 to P8.

2.2 Osteogenic differentiation

When cultured in osteogenic medium for 10–14 days, the induced hMSCs changed from spindle-shape to multiple-angled morphology and showed calcium deposit by Alizarin red staining (Figure 2(B)) compared with the control group (Figure 2(C)). At the same time, the induced cells were positive for alkaline phosphatase (ALP) staining (Figure 2(D)) and Von Kossa staining (Figure 2(E)).

2.3 Adipocyte differentiation

When cultured in adipocyte-specific induction media for 2 weeks, more than 80% of the induced hMSCs differentiated into lipid-laden cells that were stained by Oil red O (Figure 2(F)).

2.4 Endothelial differentiation

The adherent human umbilical vein endothelial cells (HUVECs) showed polygonal shapes after cultured for 4 days and typical cobblestone shapes when growing confluent under phase-contrast microscopy (Figure 3(A)). The immunofluorescent staining showed positive expression of

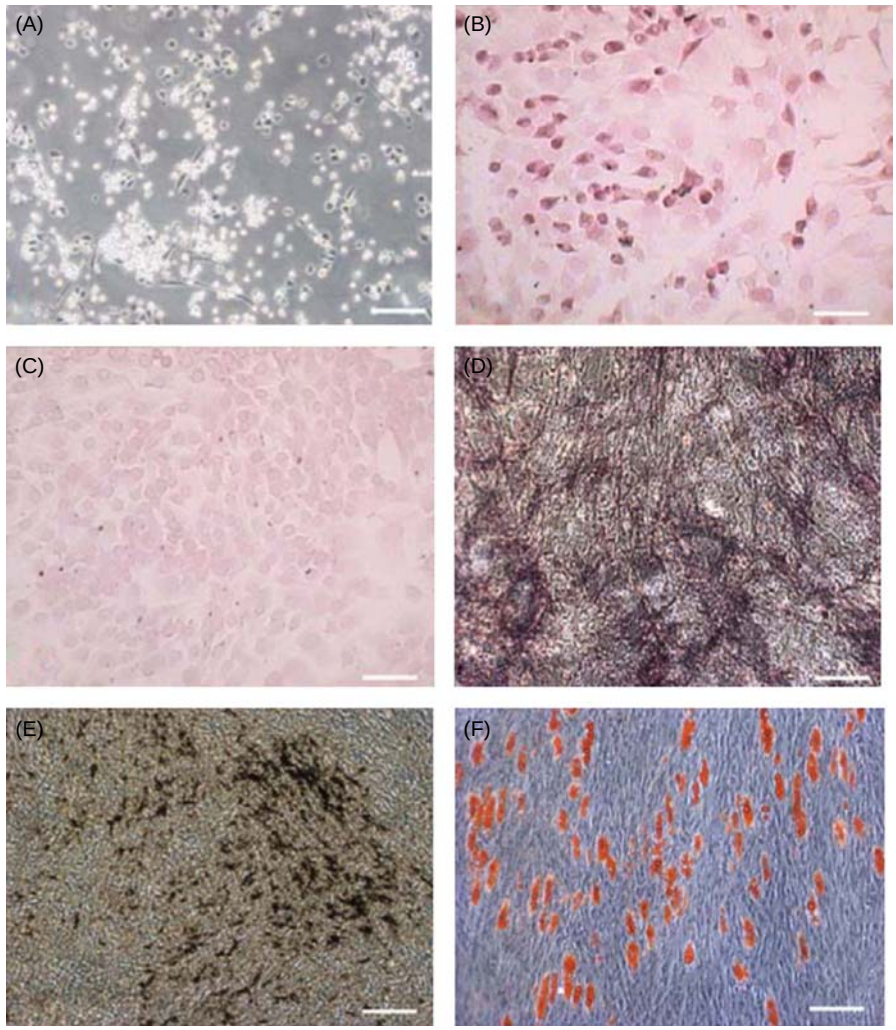

Figure 2. The culture and identification of human MSCs. (A) Attachment of single spindle hMSCs at day 3; (B) induced hMSCs showing calcium deposit after osteogenesis induction for 14 days by Alizarin red staining (arrow bars show); (C) control group; (D) ALP staining of osteogenesis induction of hMSCs after 14 days; (E) Von Kossa staining and (F) induced hMSCs showing lipoblast after 14 days by Oil red O staining (200 ×, bar = 100).

CD31 (Figure 4(A–C)), VWF (Figure 4(D–F)), and VE-cadherin (Figure 4(G–I)).

In Transwell co-culture system, the induced hMSCs showed little change after 1 week, but their bodies contracted and showed polygonal shaped morphology after 2 weeks (Figure 5(A)), compared with hMSCs as the negative control (Figure 5(B)) and HUVECs as the positive control (Figure 5(C)).

2.5 The effect of Rg1 on the differentiation of hMSCs

To test the effect of Rg1 on the differentiation of hMSCs, the specific endothelial marker CD31 expression was analyzed by flow cytometry. There was a distinguished increase in the positive CD31 expression when treated with Rg1 (from 20 to 80 μg/ml). 42.50 ± 2.62% of the induced hMSCs expressed CD31 in group Rg1

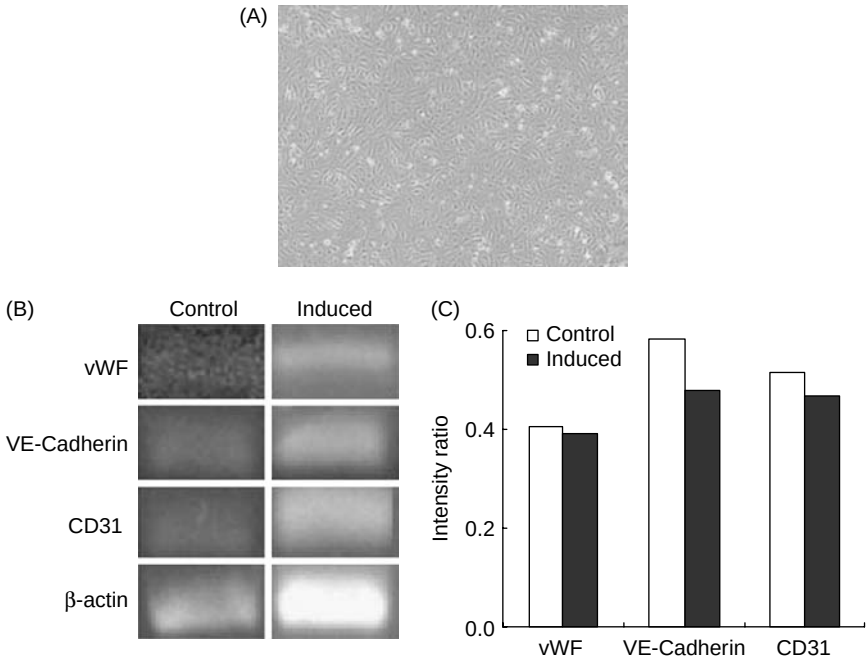

Figure 3. The morphology of HUVECs and RT-PCR analysis of the induced hMSCs and HUVECs. (A) Cobblestone shapes of HUVECs under phase-contrast microscopy. (B) The positive VWF, VE-cadherin, CD31mRNA expression of the induced hMSCs, and HUVECs (as the control group) in RT-PCR. (C) Quantification of intensity ratio for VWF, VE-cadherin, and CD31 compared to that for β-actin.

0 μg/ml, 69.34 ± 3.28% of the induced hMSCs expressed CD31 in group Rg1 20 μg/ml, 73.62 ± 2.96% of the induced hMSCs expressed CD31 in group Rg1 40 μg/ml, and 83.09 ± 4.71% of the induced hMSCs expressed CD31 in group Rg1 80 μg/ml for 3 weeks co-culture. $p < 0.05$ (Figure 6). The increased expression of the positive CD31 indicated that Rg1 promoted the milieu-dependent endothelial differentiation of hMSCs *in vitro*.

2.6 Reverse transcriptase-polymerase chain reaction analysis

The endothelial-specific markers of differentiated cells were determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Both HUVECs and differentiated hMSCs showed positive expression of CD31, VWF, and VE-cadherin mRNA (Figure 3(B)). Undifferentiated cells did not express CD31, VWF, and VE-cadherin mRNA. Compared to the HUVEC group (as the control group), the intensity ratio of CD31, VWF, and VE-cadherin mRNA PCR of the differentiated hMSC group was not statistically different ($p > 0.05$) (Figure 3(C)).

2.7 Fluorescence immunocytochemistry

The endothelial-specific markers of differentiated cells were determined by fluorescence immunocytochemistry. HUVECs and differentiated hMSCs demonstrated positive CD31 expression (Figure 5(D–F)), positive VCAM1 expression (Figure 5(G–I)), positive VE-cadherin expression (Figure 5(J–L)), and positive VWF expression (Figure 5(M–O)). Undifferentiated hMSCs did not express CD31, VCAM1, VE-cadherin, and VWF.

2.8 Electron microscopy

Electron microscopy analysis of the differentiated cells showed the typical

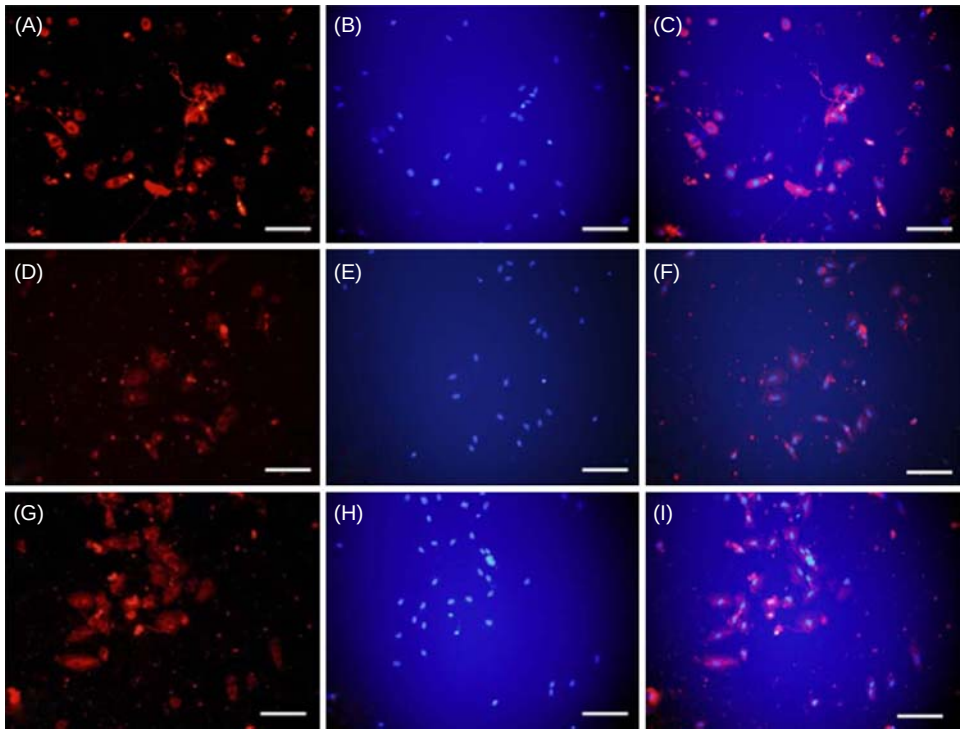

Figure 4. The immunofluorescent identification of HUVEC. (A–C) VWF factor expression of HUVECS. (A) VWF (red fluorescence), (B) Hoechst staining (blue fluorescence), and (C) overlap. (D–F) CD31 expression of HUVECs. (D) CD31 (red fluorescence), (E) Hoechst staining (blue fluorescence), and (F) overlap. (G–I) VE-cadherin expression of HUVECs. (G) VE-cadherin (red fluorescence), (H) Hoechst staining (blue fluorescence), and (I) overlap (200 ×, bar = 100 μm).

endothelial Weibel–Palade bodies (Figure 7).

3. Discussion

The present study demonstrates that ginsenoside-Rg1 significantly induces the expression of mature endothelial cell-specific markers such as CD31, VWF, and VE-cadherin in the endothelial differentiation of hMSCs co-cultured with mature endothelial cells *in vitro*.

MSCs can be differentiated into endothelial cells *in vitro* [14]. Studies also have shown that adult stem cells from bone marrow undergo milieu-dependent differentiation to express phenotypes that are similar to the cells in the local microenvironment [15,16]. The presence of various surface markers which indicate the mature endothelial phenotype is often used as a proof for endothelial differentiation. CD31 (platelet–endothelial cell adhesion molecule [PECAM]-1) is a glycoprotein expressed by endothelial cells at the endothelial cell junction where it forms Ca^{2+}-independent cell–cell adhesions. VWF is a glycoprotein of protomeric subunits derived from endothelial cells and is found circulating as multimers in a wide size range (from 0.45×10^6 to 12×10^6 Da). VE-cadherin is another major endothelial adherent junction adhesive protein. The present study showed the strong expression of CD31, VWF, and VE-cadherin mRNA by the RT-PCR analysis and protein levels by fluorescence immunocytochemistry in differentiated hMSCs co-cultured indirectly with HUVECs *in vitro*. In addition, electron microscopy showed the

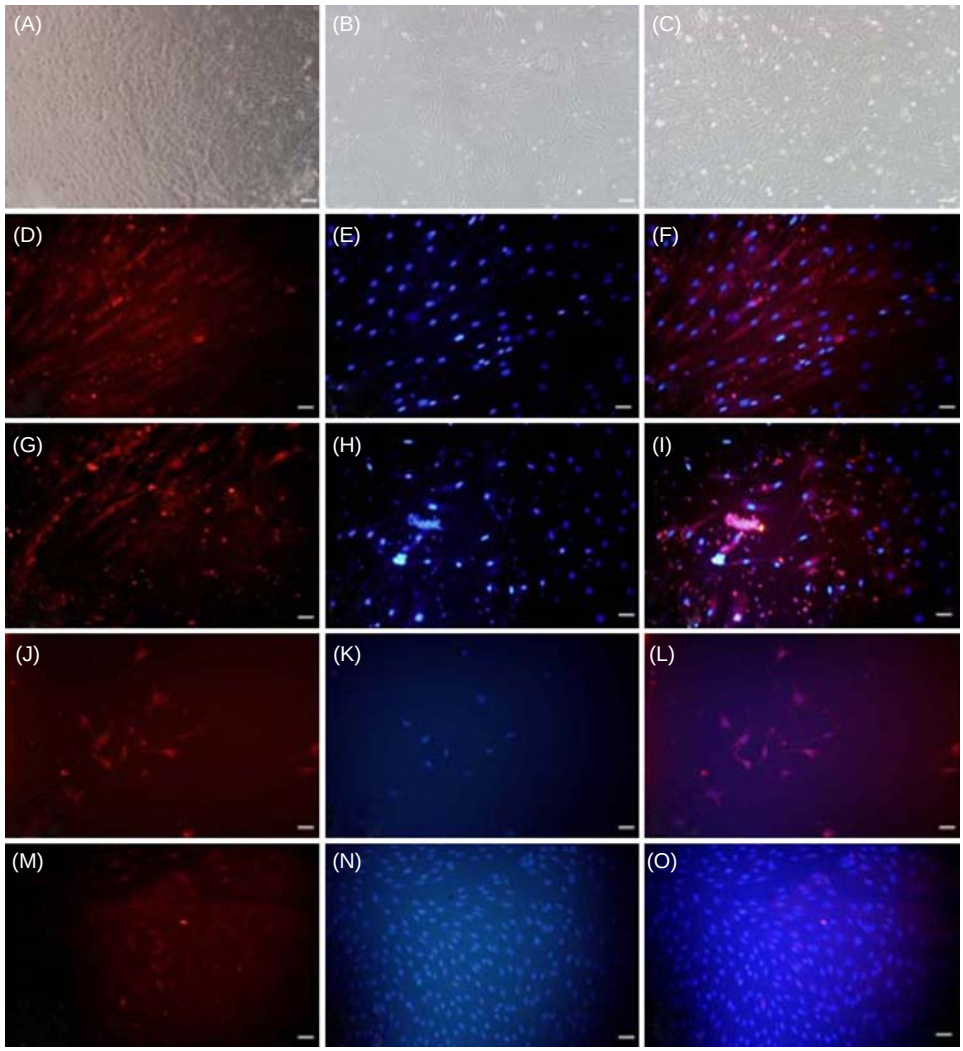

Figure 5. Endothelial morphology and immunofluorescent identification of the induced hMSCs. (A–C) The morphology identification of the induced hMSCs for 2 weeks in Transwell. (A) Induced endothelial-like cells, (B) hMSCs (as the negative control), and (C) HUVECs (as the positive control). (D–O) The immunofluorescent expression of CD31, VCAM1, VE-cadherin, and VWF of the induced hMSCs. (D) CD31 (red fluorescence), (E) Hoechst staining (blue fluorescence), (F) overlap, (G) VCAM1 (red fluorescence), (H) Hoechst staining (blue fluorescence), (I) overlap, (J) VE-cadherin (red fluorescence), (K) Hoechst staining (blue fluorescence), (L) overlap, (M) VWF (red fluorescence), (N) Hoechst staining (blue fluorescence), and (O) overlap (200 ×, bar = 100 μm).

endothelial characteristic Weibel–Palade bodies of differentiated hMSCs. These results indicated that MSCs had the milieu-dependent differentiation potential along endothelial lineage when co-cultured with mature endothelial cells.

The efficient endothelial differentiation of hMSCs could be beneficial in the successful injured vessel repair and engraftment. Panax ginseng, used for the past 4000 years in Chinese medication, has shown many clinical and pharmacological

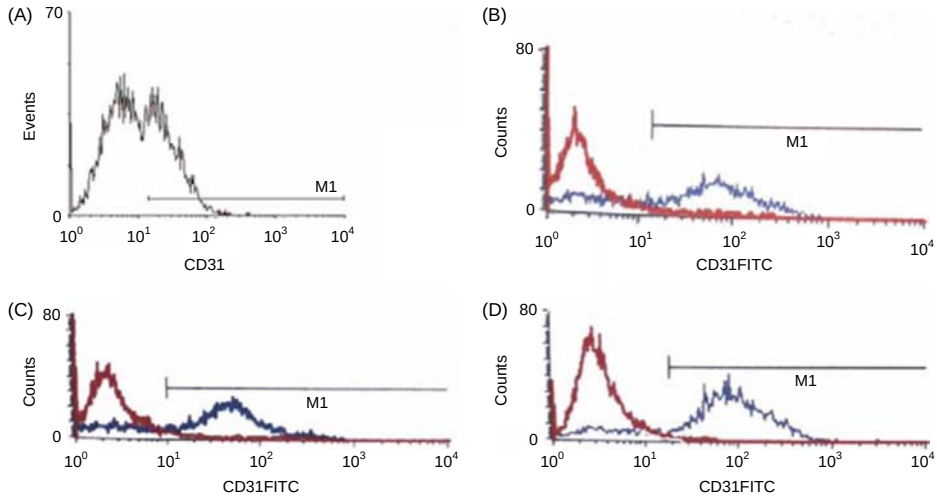

Figure 6. Flow cytometry analysis of the CD31 expression of the induced hMSCs treated by Rg1. 42.50 ± 2.62% of the induced hMSCs expressed CD31 in group Rg1 0 μg/ml, 69.34 ± 3.28% of the induced hMSCs expressed CD31 in group Rg1 20 μg/ml, 73.62 ± 2.96% of the induced hMSCs expressed CD31 in group Rg1 40 μg/ml and 83.09 ± 4.71% of the induced hMSCs expressed CD31 in group Rg1 80 μg/ml for 3 weeks co-culture ($p < 0.05$) $n = 3$.

activities. Recently, its biological effects have been investigated by molecular biological techniques. Its active components include ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, fatty acids, and mineral oils [17,18]. Of these ingredients, the ginsenosides (commonly known as saponins) exhibit a variety of cardiovascular actions including antihypertensive effect [9], protection against ischemic reperfusion injury [10], and negative chronotropic and inotropic effect [11]. Of the most active saponins, ginsenoside-Rg1 played an important role in the protective effect on injured endothelium. For example, Rg1 protected TNF-α-stimulated HUVECs by the increase in NO production [12]. As an agonist ligand for the glucocorticoid receptor (GR), Rg1 activated GR to induce rapid NO production from eNOS via the nontranscriptional PI3K/Akt pathway [19]. Rg1 also played a similar role as IL-3, IL-6, and G-CSF to enhance the proliferation of bone marrow cells [13]. In the present study, the significantly increased expression of the positive CD31 when the induced culture medium was treated with Rg1 demonstrated that Rg1 promoted the milieu-dependent endothelial differentiation of hMSCs *in vitro*. The definite mechanism is unclear now.

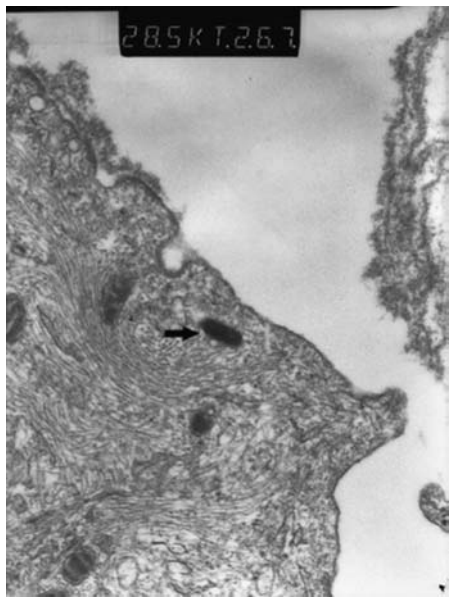

Figure 7. Electron microscopic analysis of the induced hMSCs. The characteristic Weibel–Palade body (shown by the arrow) was found in the induced hMSCs (× 28,000).

The possible explanation is that Rg1 could increase a variety of growth factors and regulating factors in the milieu-dependent differentiation of MSCs. Rg1 was shown to be a potent stimulator of vascular endothelial growth factor (VEGF) expression in HUVECs [19]. As shown in the literature [20,21], VEGF plays a pivotal role in the process of angiogenesis and vascularization. Moreover, VEGF is a part of all cocktails for the differentiation of hematopoietic stem cells into endothelial cells *in vitro* [22,23]. However, the exact mechanism needs further investigation.

In conclusion, our findings first indicate that ginsenoside-Rg1 promotes the milieu-dependent endothelial differentiation of hMSCs *in vitro*. The expression of mature endothelial cell-specific markers such as CD31, VWF, and VE-cadherin, both at mRNA and protein levels, and the endothelial characteristic Weibel–Palade body from electron microscopy further confirm that MSCs has the milieu-dependent differentiation potential along endothelial lineage.

4. Materials and methods

4.1 Chemicals

Ginsenoside-Rg1 (purity $> 99\%$) was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). A stock solution of Rg1 (10 μg/ml) was prepared in serum-free culture media. Low glucose Dulbecco's modified eagles medium (L-DMEM), Dulbecco's modified eagles medium (DMEM), and fetal calf serum (FCS) were purchased from Hyclone (Logan, IL, USA). Medium 199 (M199), bovine serum albumin, endothelial cell growth supplement (ECGS), collagenase, anti-β-actin, β-glycerophosphate, dexamethasone, ascorbic acid, isobutylmethylxanthine, hydrocortisone, indomethacin, Oil red O solution, and saponin were purchased from Sigma (Santa Clara, CA, USA). Penicillin/streptomycin was purchased from Gibco (New York, USA). Trypsin was purchased from Amresco (Solon, OH, USA). Fermentas RT-PCR kit, Trizol, and Taq were purchased from Invitrogen (Camarillo, CA, USA). FITC-anti human CD44, CD105, CD31 PECAM-1, PE-CD29, CD34, CD11b, and PerCP-CD45 were purchased from Pharmingen (San Diego, CA, USA). Anti-VWF was purchased from Dako (Copenhagen, Denmark). VE-cadherin was purchased from Santa (Santa Cruz, CA, USA).

4.2 Isolation and culture of human bone marrow-derived MSCs

Human bone marrow samples were collected from patients undergoing selective vertebrate orthomorphia after obtaining informed consent according to procedures approved by the Ethics Committee at Guangdong General Hospital. Bone marrow aspirate diluted in phosphate-buffered saline (PBS) was layered over a Percoll solution ($d = 1073$ g/ml) slowly and then centrifuged through a density gradient to eliminate unwanted cell types. Mononuclear cells at interface were seeded into 75 cm^2 flasks containing L-DMEM supplemented with 10% FCS and grown at 37°C in 5% CO_2. Non-adherent cells were removed after 3 days by medium change. The medium was changed subsequently every 4 days. When the culture reached 90% confluence, MSCs were removed using 0.25% Trypsin and replated at a 1:3 dilution under the same culture conditions. All the experiments were performed with the passages 3–8.

4.3 Osteogenic differentiation

The culture-expanded cells at 2×10^4 cm^{-2} were induced in the following osteogenic medium for 2–3 weeks: DMEM supplemented with 10% FCS, 10 mmol/L β-glycerophosphate, 10^{-7} mol/L dexamethasone, and 0.2 mmol/L ascorbic acid. Then, the cells were stained with von Kossa to reveal osteogenic differentiation.

4.4 Adipogenic differentiation

The culture-expanded cells at $2 \times 10^4 cm^{-2}$ were induced for 3 weeks in DMEM supplemented with 10% FCS, 0.5 μmol/L hydrocortisone, 0.5 mmol/L isobutylmethylxanthine, and 50 μg/ml indomethacin. At the end of the culture, the cells were fixed in 10% formalin for 10 min and stained with fresh Oil red O solution to show lipid droplets in induced cells.

4.5 Culture of HUVECs

HUVECs were collected from patients undergoing cesarean section at Guangdong General Hospital, after obtaining informed consent. HUVECs were harvested from fresh umbilical cords by collagenase treatment as previously described [24]. The cells were grown in M199 containing 20% FCS, 75 μg/ml ECGS, 100 U/ml heparin, and 1% penicillin/streptomycin. Cultures were maintained at 37°C in humidified CO_2 incubator (5% CO_2). HUVECs at passages 2–5 were used in the experiments. Monolayers were identified as endothelial cells by RT-PCR analysis and periodically by immunofluorescent staining for CD31, VWF, and VE-cadherin.

4.6 Endothelial differentiation and Rg1 treatment

Indirect co-cultures were established using Transwell insert membrane (with pores of 0.4 μm in diameter, Corning, NY, USA). HUVECs and hMSCs were suspended in M199 supplemented with 20% FCS, 100 U/ml heparin, 50 μg/ml ECGS and planted in the upper and lower chambers, respectively, of six-well plates (at ratios of 5×10^4:5×10^3 cells/well). Co-cultures were incubated at 37°C in 5% CO_2 incubator. The medium was changed every 3 days. HUVECs were replaced every week to keep 90% confluence. To test the effect of Rg1 on the differentiation of hMSCs, we added the indicated doses 0, 20, 40, and 80 μg/ml of Rg1 to the co-culture system for 3 weeks, respectively. The positive CD31 expression percentage of the induced cells treated by Rg1 was analyzed by flow cytometry. The endothelial phenotype of the induced cells was analyzed from mRNA expression, fluorescence immunocytochemistry, and microstructure. The undifferentiated hMSCs were used as negative control and HUVECs were used as positive control for the experiments.

4.7 RT-PCR analysis

Mature and functional endothelial markers VWF, CD31, and VE-cadherin mRNA expressions were determined by RT-PCR. Total cellular RNA was extracted from 5×10^4 HUCECs and differentiated hMSCs. The same amount of total RNA was treated with Fermentas RT-PCR kit to generate cDNA using an oligo (d*T*) adaptor primer. Then, PCR amplification was performed for human VWF, CD31, and VE-cadherin. The primers used are listed in Table 1. PCR cycles were as follows: 94°C for 5 min, 95°C for 30 s, 55–62°C

Table 1. Primer sequences of endothelial markers used for RT-PCR.

Primer	Sequence	Size (bp)	T_m (°C)
VWF	5-GCTCTCTCTCTTACCCGGATG-3 5-AGGTAGTCCCGTTCCTCATA-3	556	55
CD31	5-TCCGATGATAACCACTGCAA-3 5-GTGGTGGAGTCTGGAGAGGA-3	297	58
VE-cadherin	5-CCACATTCAGGGAAATGCTT-3 5-GAACATCTGCCCCTTCTCAG-3	201	56
β-Actin	5-GTG GGG CGC CCC AGG CAC CA-3 5-CTC CTT AAT GTC ACG CAC GAT TTC-3	540	62

(depending on the primer set) for 30 s, and 72°C for 60 s (40 cycles), 72°C for 10 min. The RT-PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized with ethidium bromide.

4.8 Fluorescence immunocytochemistry

The endothelial-specific markers VWF, CD31, and VE-cadherin expressions of the induced cells were analyzed by fluorescence immunocytochemistry. Cells were fixed with normal goat serum for VWF and 2% normal donkey serum for 60 min at room temperature and incubated with primary antibody (rabbit-anti-human VWF multiclonal antibody 1:250, goat anti-human CD31, VE-cadherin monoclonal antibody 1:5) overnight at 4°C. The cells were rinsed with 0.01 M PBS three times and then incubated with PE-conjugated goat anti-rabbit IgG (H + L, 1:150) and CY3-conjugated donkey anti-goat IgG (H + L, 1:150) for 45 min at room temperature. Cell nuclei were stained with 1 μg/ml Hoechst33344. Samples were examined under fluorescence microscopy.

4.9 Electron microscopy

The microstructure of the induced cells was observed by electron microscopy. To detect the endothelial-specific Weibel–Palade bodies, we washed the $2 \times 10^6\,ml^{-1}$ differentiated hMSCs with PBS, fixed in 3% paraformaldehyde for 1 h at 4°C, and then observed under electron microscopy after dehydration, embedding, and slicing.

4.10 Flow cytometry of induced MSCs

The percentage of the endothelial-specific marker CD31 expression was analyzed by flow cytometry after the induced cells were treated by Rg1. The cells were trypsinized, washed with PBS containing 0.5% bovine serum albumin, and incubated with antibodies against human CD31. The suspended cells were analyzed by FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA) and the CellQuest Pro software (BD Biosciences, San Jose, CA, USA).

4.11 Statistical analysis

All experiments were repeated at least for three times. Values were depicted as mean ± SEM. One-way ANOVA was used for statistical analysis of the original data, and significance was accepted at $p < 0.05$.

Acknowledgements

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